

## APPENDIX - 1

### EXTRACTION OF ALGAL DNA

#### (Kit method)

1. The powdered algal sample was homogenized in 1ml of extraction buffer (200mM Tris HCL, pH 7.5, 25mM EDTA, 250mM NaCl and 0.5% SDS) and left at room temperature for 30min. After 30min of incubation time, the contents were centrifuged and the supernatant was collected.
2. To this equal volume of ice cold Phenol: Chloroform (1:1) was added and the contents were mixed and centrifuged at 4000rpm for 6min.
3. The supernatant was collected and 300µl of chilled chloroform was added. The content was mixed well thoroughly by inverting the tube repeatedly. The mixture was again centrifuged at 4000rpm for 6min.
4. Then 300µl of ice cold iso-propanol was added and the content was mixed gently by inverting the tube, kept at -20<sup>0</sup>C for 30min and centrifuged at 4000rpm for 6min.
5. The supernatant was discarded and the pellet was washed with 100µl of ice cold 70% ethanol and centrifuged at 4000rpm for 6min. The pellet was dried and dissolved in 30µl of TE buffer (pH 8.0) and 1-5µl of the isolated DNA was used for PCR amplification using the primers A1 and A2.

## APPENDIX - 2

### PURIFICATION OF ALGAL DNA

#### (Qiaquick gel extraction kit)

This protocol is for the purification of DNA up to 10 µg (70 bp to 10 kb). The yellow colour of buffer QG (yellow colour) indicates pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5. Ethanol (96–100%) was added to buffer PE before use. Isopropanol (100%) and a heating block or water bath at 50°C are required. The centrifugation process was carried out at 17,900 x g (13,000 rpm) in a conventional table-top micro centrifuge.

1. The isolated DNA was subjected to 1% agarose gel electrophoresis and viewed under UV-transilluminator.
2. The DNA fragment from the agarose gel was excised with a clean, sharp scalpel and weigh the gel.
3. Added thrice the volume of buffer QG to 1 volume gel (100 mg gel~100µl). (The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG).
4. Incubated at 50°C for 10 min (or until the gel slice was completely dissolved) and vortex the tube for every 2-3 min to dissolve the gel. After the gel slice has dissolved completely, the colour of the mixture was checked (yellow) (similar to buffer QG without dissolved agarose). If the colour of the mixture is orange or violet, added 10µl of 3M sodium acetate (pH 5.0) and mixed well until the mixture turns yellow.
5. Added 1 gel volume of isopropanol to the sample and mixed well.
6. Placed a QIAquick spin column in the 2 ml collection tube or into a vacuum manifold. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min or applied vacuum to the manifold until all the samples have passed through the column. Discarded the flow-through and placed the QIAquick column back into the same tube (For sample volumes of >800 µl, load and spin/apply the vacuum again).
7. If DNA is subsequently used for sequencing *in vitro* transcription microinjection, 500 µl of buffer QG was added to the QIAquick column and centrifuged for 1 min or apply vacuum. Discarded the flow-through and placed the QIAquick column back into the same tube.
8. Added 750 µl buffer PE to QIAquick column and centrifuged at 1 min or apply vacuum. Discarded the flow-through and placed the QIAquick column back into the same tube. Centrifuged the QIAquick column in the provided 2ml collection tube for 1 min to remove the residual wash buffer.
9. Placed QIAquick column into a clean 1.5 ml micro centrifuge tube.
10. To elute DNA, added 50µl of buffer EB (10 mM Tris·Cl, pH 8.5) or water to the centre of the QIAquick membrane and centrifuged the column for 1 min. For increased DNA concentration, added 30µl of buffer EB to centre of the

QIAquick membrane, let the column stand for 1 min and then centrifuged for 1 min. After the addition of buffer EB to the QIAquick membrane, increase the incubation time was increased upto to 4min which can increase the yield of the purified DNA.

11. If purified DNA is to be analysed on a gel, added 1 volume of loading dye to 5 volumes of purified DNA. Mixed the solution by pipetting up and down before loading the gel.

### APPENDIX - 3

#### DETERMINATION OF PH

(Jackson, 1973)

##### PRINCIPLE

A glass surface in contact with hydrogen ions of the solution under test, acquires an electrical potential which depends on the concentration of hydrogen ions. A measure of the electrical potential (emf), gives hydrogen ion concentration or pH of the solution.

##### PROCEDURE

The pH was standardised using buffer solutions of pH 7.0 to 9.2. After standardisation the pH was determined by the pH meter. 20 g of the air dried soil and algal compost samples were passed through 2 mm sieve, transferred to a clean 100 ml beaker and 50 ml of distilled water was added. The contents were stirred intermittently using glass rod and after 20 minutes the electrodes were washed with distilled water, wiped with filter paper and the electrodes were immersed in soil and algal compost water suspension separately and the readings were recorded respectively. Similarly the pH meter was dipped in effluent samples to record the pH values.

### APPENDIX - 4

#### DETERMINATION OF ELECTRICAL CONDUCTIVITY

(Jackson, 1973)

##### PRINCIPLE

Electrical conductivity represents the total amount of soluble salts present in the soil, algal compost and in the effluent samples.

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**PROCEDURE**

The electrical conductivity was measured in terms of the resistance offered to the flow of current using a conductivity bridge and expressed in mmhos / cm. the conductivity bridge was immersed in soil, algal compost water suspension and the effluent samples separately to record the EC.

**APPENDIX - 5****ESTIMATION OF AVAILABLE NITROGEN  
ALKALINE POTASSIUM PERMANGANATE METHOD  
(Subbaih and Asija, 1956)****PRINCIPLE**

The amount of nitrogen released from the soil and algal compost by the potassium permanganate oxidation of a part of soil compost organic matter is estimated by distillation with sodium hydroxide. The distillate is collected in boric acid containing double (mixed) indicator and titrated against standard sulphuric acid.

**REAGENTS**

0.32% potassium permanganate, 2% boric acid and 2.5 % sodium hydroxide, Double (mixed) indicator: Bromocresol green (0.5 g) and methyl red, (0.01 g) were dissolved in 100 ml of ethyl alcohol, Standard sulphuric acid (N / 50).

**PROCEDURE**

Twenty grams of soil and algal compost was weighed separately and transferred to a distillation flask. To this, 20 ml of distilled water and 1 ml of liquid paraffin (to control frothing) were added. A few glass beads (with holes) were added to prevent bumping. 100 ml of 2.32% potassium permanganate and 2.5% sodium hydroxide solutions were added to the distillation flask. The contents were distilled and the liberated ammonia was collected in 500 ml ice tumbler containing 20 ml of boric acid with double mixed indicator. The distillation was continued, for 30 minutes until 100 ml of distillate was collected in the beaker. The ammonia collected was titrated against the standard sulphuric acid (N/50) and from the titre value obtained available nitrogen content in the soil and algal compost were calculated respectively.

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**APPENDIX - 6****ESTIMATION OF AVAILABLE PHOSPHORUS****FLAME PHOTOMETRIC METHOD****(Olsen *et al.*, 1954)****PRINCIPLE**

Phosphorus is extracted from the soil and algal compost with 0.5M NaHCO<sub>3</sub> and adjusted to pH 8.5. The CO<sub>3</sub><sup>2-</sup> ions from NaHCO<sub>3</sub> will react with Ca<sup>2+</sup> and forms CaCO<sub>3</sub> in the solution which develops blue colour by ascorbic acid method. The amount of phosphorus extracted was measured colorimetrically.

**REAGENTS**

1. 0.5 N sodium bicarbonate (pH adjusted to 8.5) - 42 g of NaHCO<sub>3</sub> was dissolved in one litre of distilled water. Activated carbon (free of phosphorus by washing with 0.5 M sodium bicarbonate)
2. Reagent A: 12 g of ammonium molybdate was dissolved in 250 ml of distilled water. 0.291g of antimony potassium tartarate was dissolved in 100 ml distilled water. Both the solutions were added to 1000 ml of 5N sulphuric acid (13mL of sulphuric acid added to 100ml of distilled water). The above contents were mixed thoroughly and made up to 2 litres with distilled water.
3. Reagent B: 1.056 g of ascorbic acid was dissolved in 200 ml of reagent A. The solution was prepared freshly as and when required.

**PROCEDURE**

Five gram of soil and algal compost was weighed and transferred to a 100 ml polyethylene shaking bottle (carried out a blank side by side) to which 50 ml of 0.05M NaHCO<sub>3</sub> (pH - 8.5) and a pinch of activated carbon (to make the extracted solution colourless) was added. The above contents were mixed in a reciprocating mechanical shaker for 30 minutes, filtered through Whatman number 4 filter paper and the filtrate was collected in a clean dry beaker (The filtrate should be clean and colourless). Five ml of the filtrates were pipetted in a 25 ml volumetric flask, 4ml of reagent B was added and made up the volume to 25ml. The contents were allowed to stand for 10 minutes and the colour development was measured. The intensity of

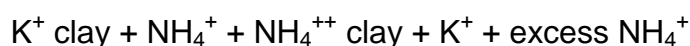
the blue colour developed was read in a photoelectric colorimeter using a red filter (660 nm wave length) by adjusting the meter to 100 per cent transmittance with the blank. From the standard curve, the concentrations of phosphorus was calculated in the solution against the per cent transmittance observed in the above step.

## APPENDIX - 7

### ESTIMATION OF AVAILABLE POTASSIUM AND SODIUM FLAME PHOTOMETRIC METHOD (Olsen *et al.*, 1954)

#### PRINCIPLE

The potassium ( $K^+$ ) and sodium (Na) ions in the exchange sites are replaced with ammonium ( $NH_4^+$ ) ions leached from the soil and algal compost. The reaction may be illustrated as follows:



The reaction goes to the right because of excess ammonium ions as shown by  $NH_4$  also being present on the right side of the equation. The  $K^+$  and  $Na^+$  ions in solution were determined using the flame photometer.

#### REAGENTS

1. Neutral normal ammonium acetate
2. Standard potassium solution

#### PROCEDURE

Five gram of the soil and algal compost samples were taken in a 100 ml polythene shaking bottle separately. 25 ml of neutral normal ammonium acetate was added and kept in a mechanical reciprocating shaker for 5 minutes. The contents were filtered through dry Whatman No. 40 filter paper and the filtrates were collected in a dry test tube. The amount of potassium and sodium in the filtrates were measured in the flame photometer. From the concentration of potassium and sodium read in the standard curve corresponding to the flame photometer reading, the amount of available potassium and sodium in the soil and algal compost was calculated separately.

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**APPENDIX - 8****ESTIMATION OF AVAILABLE CALCIUM****EDTA METHOD****(Subbaih and Asija, 1956)****PRINCIPLE**

Ca and Mg ions form stable complex by EDTA at different pH. Calcium is estimated by murexide indicator at pH 12 in the presence of NaOH. Then Ca+Mg is estimated using eriochrome black T indicator at pH 10 in the presence of  $\text{NH}_4\text{Cl}$  and  $\text{NH}_4\text{OH}$  buffer solution and titrated against EDTA (versenate).

**REAGENTS****4 N sodium hydroxide**

160 g of NaOH was dissolved in 1 litre of water.

**Standard calcium chloride solution (0.01 N)**

To 0.5 g of pure calcium carbonate, 10 ml of 3 N HCl was added. The contents were boiled to expel carbondioxide and then made up to 1 litre with distilled water.

**Ammonium purpurate (Murexide) indicator**

0.5 g of ammonium purpurate was mixed with 100 g of powdered potassium sulphate (This is not stored in the form of solution as it gets oxidised).

**Ethylene diamine tetra acetic acid (Versenate) solution (0.01 N)**

Two grams of disodium hydrogen ethylene diamine tetraacetate and 0.05 g of magnesium chloride hexahydrate was dissolved in distilled water and made up to 1 litre. The solutions were titrated against calcium chloride using the indicators separately for calcium and magnesium.

**PROCEDURE**

To 5 g of air dried soil, 25 ml of neutral normal ammonium acetate was added, placed it on a shaker for 5 minutes and filtered it through Whatman filter paper No.1. Calcium and magnesium concentration was determined after pretreatment.

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## PRETREATMENT OF SOIL SAMPLE

Ammonium acetate extract was transferred to 250ml conical flask and evaporated to dryness on a hot plate. One millilitre of nitric acid and 3ml of hydrochloric acid was added and evaporated again. The residue was dissolved after evaporation in 20ml of 0.1N acetic acid, filtered and diluted to 100ml.

## DETERMINATION OF CALCIUM

Five millilitre of the above aliquot was pipetted and diluted to 25 ml. To this 0.25 ml of 4 N sodium hydroxide and 50 mg of ammonium purpurate indicator was added and titrated with 0.01 N EDTA until the colour change was observed from orange red to purple and the total amount of calcium was calculated

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## APPENDIX - 9

### ESTIMATION OF AVAILABLE MICRONUTRIENTS IN SOIL

Lindsay and Norvell (1978)

DTPA (Diethylene triamine pentaacetic acid) was used as an extractant which has been widely accepted for the simultaneous extraction of micronutrient cations viz. Zinc (Zn), Copper (Cu), Iron (Fe) and Manganese (Mn) in neutral and alkaline soil. The content of these cations in the extract was determined on an Atomic Absorption Spectrophotometer (AAS).

### PRINCIPLE

DTPA, a chelating agent combines with free metal ions in the solution to form soluble complexes. The stability constants for simultaneous complexing of Zn, Cu, Mn and Fe show DTPA as a most suitable extractant. The DTPA has a capacity to complex each of the micronutrient cations as 10 times of its atomic weight.

### REAGENTS

#### 1. Extracting solution

0.005M DTPA, 0.01M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 0.1M TEA (Triethanolamine) was adjusted to pH 7.3. 1.967g DTPA and 13.3ml TEA was dissolved in 100ml of deionized distilled water. 1.47g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was added and the volume was

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made upto 500 ml with distilled water. DTPA - TEA mixture was added to it and the final volume was made up to 900 ml. The pH was adjusted to 7.3 using 1 N HCl and made the final volume to one litre and mixed thoroughly.

## 2. Working standard solutions

Zinc : 0.439g of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$  was dissolved in 5ml of 1:5 sulphuric acid and made up to 1000ml with distilled water. 2, 4, 6 and 8 ml of stock solution (10 $\mu\text{g}$  Zn/ml) was taken and diluted to 100 ml using DTPA extracting solution, which gives the concentration of 0.2, 0.4, 0.6 and 0.8  $\mu\text{g}$  / ml of zinc.

Iron : 0.702g of ferrous ammonium sulphate hexahydrate was dissolved in 5ml of 1:5 sulphuric acid and made up to 1 litre with distilled water. 1, 2, 4, 6 and 8 ml of stock solution (100 $\mu\text{g}$ ) was taken and diluted to 100 ml with DTPA extracting solution which gives the concentration of 2, 4, 6 and 8 $\mu\text{g}$ /ml of iron.

Copper : 0.392g of  $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$  was dissolved in 5ml of 1:5 sulphuric acid and made upto 1000ml with distilled water. 2, 4, 6 and 8 ml of stock solution containing 100 $\mu\text{g}$  Cu/ml was made up to 100ml with DTPA extracting solution which gives the concentration of 2, 4, 6 and 8 $\mu\text{g}$ /ml of Cu.

Manganese : 0.288g of  $\text{KMnO}_4$  was dissolved in 300 ml of distilled water. To this, 20ml of concentrated sulphuric acid was added and made up to 1000 ml with distilled water. About 2, 4, 6 and 8 ml of stock solution containing 100 $\mu\text{g}$  Mn/ml was made up to 100ml with DTPA extracting solution which gives a concentration of 2, 4, 6 and 8 $\mu\text{g}$  /ml of Mn.

## PROCEDURE

10g of air dried soil sample was weighed in a polythene bottle and 20 ml of the DTPA - extracting solution was added. The bottles were closed and kept on an shaker for two hours, filtered and the filtrate was analysed for Zn, Cu, Fe and Mn with the atomic absorption spectrophotometer. From the standard curve the concentrations of micronutrients were calculated.

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**APPENDIX - 10****ESTIMATION OF NICKEL  
DIMETHYL GLYOXIME COLORIMETRIC METHOD  
APHA (1998)****PRINCIPLE**

Nickel reacts with dimethyl glyoxime in the presence of an alkaline oxidising agent to form a characteristic red colour complex which can either be measured visually or photometrically.

**INTERFERENCES**

Iron, manganese and copper interfere and if they are present in concentration thrice that of nickel, their interference is suppressed by the addition of sodium citrate. If they are present in excess concentrations, nickel dimethyl glyoxime complex alone is separated by extraction with chloroform and preceded further.

**REAGENTS**

Nickel stock solution : 447.9 mg nickel sulphate was dissolved in distilled water and made up to 1000 ml in a volumetric water (1 ml = 10 $\mu$ g Ni).

Nickel working solution : 10ml of nickel stock solution was pipetted into a 100 ml volumetric flask and made up to the mark with distilled water (1 ml = 10  $\mu$ g Ni).

Hydrochloric acid, 0.5N : 43ml of conc. HCl was diluted to 1000 ml with distilled water.

Sodium citrate solution : 125g of sodium citrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> · 2H<sub>2</sub>O) was dissolved in 500 ml of distilled water.

Iodine solution, 0.05 N : 20g of potassium iodide was dissolved in 50 ml of distilled water and to this 6.4 g of iodine was added and made up to 1000 ml.

Dimethyl glyoxime solution, 0.5 % : 1g of dimethyl glyoxime was dissolved in 100ml of concentrated ammonia solution, 100ml distilled water was added and filtered.

Additional reagents for the removal of interferences:

1. Dilute ammonia solution : 10 ml of concentrated ammonia solution was diluted to 450 ml with distilled water.
2. Chloroform
3. 4 ml of dimethyl glyoxime solution

## PROCEDURE

Nickel working solution covering the range up to 100 µg was taken in a series of 50ml Nessler tubes and distilled water was kept as the blank. One millilitre of the neutralised (acid digested sample containing not more than 100 µg nickel) sample was taken in a 50ml Nessler tube. To the blank, standards and samples, 20 ml of 0.5 N HCl was added. Then the following reagents were added in order with mixing after each addition:

- 10 ml sodium citrate solution
- 2 ml iodine solution
- 4 ml dimethyl glyoxime solution

The volume in all the flasks were made up to 50 ml with distilled water and allowed to stand for 20 minutes. Optical density was measured in a spectrophotometer at 470 nm against the reagent blank. A calibration curve was prepared and the microgram of nickel equivalent to the observed optical density was determined and the results were expressed.

## APPENDIX - 11

### ENUMERATION OF RED BLOOD CORPUSCLES

#### DILUTING FLUID METHOD

Rusia and Sood (1992)

#### PRINCIPLE

The blood specimen is diluted to 1:200 dilutions with RBC diluting fluid which does not remove the white cells but allows the red cells to be counted under high power (40x) objective using a Neubauer's counting chamber. The number of cells in undiluted blood is calculated as the number of red cells per cubic mm of whole blood.

#### REQUIREMENTS

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Neubauer's counting chamber, RBC pipette, RBC diluting fluid and EDTA blood

### **PROCEDURE**

Blood was drawn into the RBC pipette up to 0.5 graduation mark. It was followed immediately by drawing RBC diluting fluid up to 101 mark and mixed thoroughly. One drop of diluted blood was released into the narrow slit between the counting chamber and the cover slip. The fluid spreads by capillary action. The cells were allowed to settle to the bottom of the chamber for 2 minutes. The chamber was placed on the stage of the microscope and focused under low power followed by high power and counting of RBC is noted. Erythrocytes were counted in the 5 squares of the counting area of 1 mm square. The total number of cells in 16 squares is multiplied by 10,000 to give the number of cells in millions/mm<sup>6</sup> blood.

## **APPENDIX - 12**

### **DETERMINATION OF PACKED CELL VOLUME**

**Nelson and Morris (1989)**

#### **PRINCIPLE**

Anticoagulated whole blood is centrifuged and the volume occupied by the erythrocytes is expressed as percentage of the total volume of whole blood.

#### **REQUIREMENTS**

Micro-haematocrit tubes, Micro-haematocrit centrifuge and Micro-haematocrit reader

#### **PROCEDURE**

Blood sample was collected in a test tube containing EDTA and was mixed well gently for 2 minutes. The well-mixed blood was drawn up a capillary tube for 1/2 to 3/4 of its length. One end of the tube was sealed with sealing wax or plasticine and placed in the micro-haematocrit centrifuge. The tubes were centrifuged at 11,000 rpm for 5 minutes and read on the reader. The reading was expressed as percentage of packed red cells in the total volume of whole blood.

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**APPENDIX - 13****ESTIMATION OF HAEMOGLOBIN  
SPECTROPHOTOMETRY- CYANMETHEMOGLOBIN METHOD  
(Drabkin, 1946)****PRINCIPLE**

Hemoglobin in the presence of potassium ferricyanide at alkaline pH is oxidized to methemoglobin. The methemoglobin formed reacts with potassium cyanide to yield a red coloured complex cyanmethemoglobin which is measured spectrophotometrically at 540 nm.

**REAGENTS**

Drabkins solution- Dissolved 50 mg of potassium cyanide, 20 mg of potassium ferricyanide and 1 g of sodium bicarbonate in distilled water and made up the volume to 1 liter in a volumetric flask. The reagent was stored in an amber coloured reagent bottle.

Standard Hemoglobin solution- 61 mg of hemoglobin was dissolved in 100 ml of distilled water.

**PROCEDURE**

5 ml of Drabkins solution was pipetted into three test tubes labeled as blank, standard and test. 0.02 ml of distilled water, standard hemoglobin and whole blood were added to the respective tubes. All the tubes were mixed well and kept at room temperature for 5 minutes. The absorbance of the standard and test sample was read at 540 nm against the reagent blank in a spectrophotometer.

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**APPENDIX - 14****ENUMERATION OF WHITE BLOOD CORPUSCLES  
TRUCK'S FLUID METHOD  
Rusia and Sood (1992)****PRINCIPLE**

Blood is diluted with weak acid solution which removes the red cells by hemolysis and also accentuates the nuclei of the white cells thus making the counting of the white cells easy. The number of white cells per cubic mm in undiluted

whole blood is calculated by knowing the volume of fluid examined and the dilution of the blood.

## REQUIREMENTS

Neubauer's counting chamber, WBC pipette, WBC diluting fluid and EDTA blood

## PROCEDURE

Blood was sucked upto 0.5 marks in WBC diluting fluid. WBC diluting fluid was sucked in the same pipette upto mark 11. Contents of the pipette were then mixed for 3 minutes by rotating the pipette at an angle of  $45^{\circ}$  and after 2 minutes time the leucocytes were counted in four large corner squares under the microscope.

## APPENDIX - 15

### CALCULATION OF ERYTHROCYTE INDICES

Blaxhall and Daisly (1973)

#### Mean Cell Volume (MCV)

The MCV is the average volume of red cells and is calculated from the haematocrit (HCT) and red cell count (RBC).

$$\text{MCV (cubic micra)} = \frac{\text{HCT (\%)}}{\text{RBC (millions /cu.mm x 106)}} \times 100$$

#### Mean Cell Haemoglobin (MCH)

The MCH is the content (weight) of hemoglobin of the average red cell. It is calculated from the hemoglobin concentration and the red cell count

$$\text{MCH (picograms)} = \frac{\text{Hb (g/dl)}}{\text{RBC (millions /cu .mm) x 106}} \times 10$$

#### Mean Cell Haemoglobin Concentration (MCHC)

The MCHC is the average concentration of hemoglobin in a given volume of packed red cells. It is calculated from the hemoglobin concentration and the hematocrit.

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl)}}{\text{HCT (\%)}} \times 100$$

### APPENDIX - 16

#### ESTIMATION OF GLUCOSE IN PLASMA SPECTROPHOTOMETRY GLUCOSE OXIDASE/PEROXIDASE METHOD (Trinder, 1969)

##### PRINCIPLE

Glucose is oxidized by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of peroxidase (POD) oxidizes the chromogen 4-aminopyrine/phenolic compound to a red coloured compound. The intensity of the red coloured compound is proportional to the glucose concentration and is measured at 505 nm.

##### Reagents

Reagent 1 (Buffer / Enzymes / Chromogen): The reagent was reconstituted with distilled water.

Standard: 100 mg% glucose

##### PROCEDURE

10 µl of distilled water, glucose standard and plasma were pipetted into three clean dry test tubes labeled blank, standard and test. 1.0 ml of enzyme reagent was added to all the test tubes, mixed well and incubated at 37°C for 15 minutes. The colour developed was measured at 505 nm and the amount of glucose present in the plasma was calculated

### APPENDIX - 17

#### ESTIMATION OF TOTAL PROTEIN PLASMA SPECTROPHOTOMETRY (Doumas *et al.*, 1971)

##### PRINCIPLE

Peptide bonds of proteins react with copper ions in alkaline medium to give a violet coloured complex. The intensity of this colour is proportional to the protein concentration which is measured at 555 nm.

**REAGENTS**

Biuret reagent and Total protein standard: 5.5 g%

**PROCEDURE**

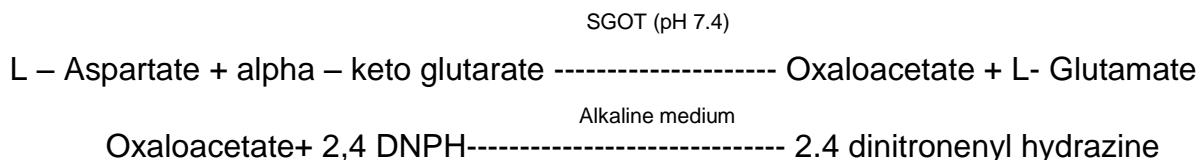
50µl of distilled water, protein standard and plasma were pipetted into three clean dry test tubes labeled blank, standard and test. 1.0 ml of biuret reagent and 2 ml of distilled water were added to all the test tubes, mixed well and incubated at 37°C for 5 minutes. The absorbance of test and standard was read against reagent blank at 555 nm and the amount of plasma protein was calculated.

**APPENDIX - 18****ESTIMATION OF ASPARTATE AMINO TRANSAMINASE (AST)**

Reitman and Franckel (1957)

**PRINCIPLE**

Serum glutamine oxaloacetic transaminase catalyses the reversible transfer of an amino group from aspartate to alpha-keto glutarate forming glutamate and oxaloacetate SGOT catalyses the following reaction

**Reagents**

1. Tris buffer, pH 7.5 – 100mmol/l
2. L. aspartate – 500mmol/l
3. 2-oxoluglutarate – 15mmol/l
4. 2,4, dinitronenyl hydrazine reagent
5. Working sodium hydroxide (4N)

**Procedure**

0.5ml of buffered substrate was incubate at 30<sup>0</sup>C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37<sup>0</sup>C dor 30minutes. Then 0.5ml of

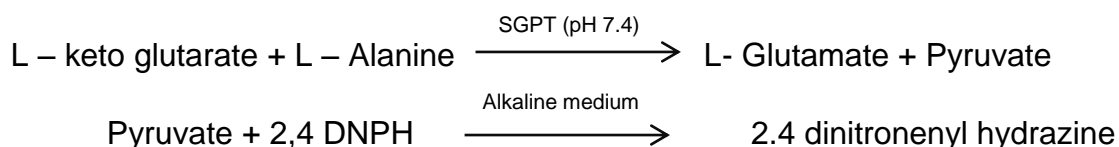
2, 4 dinitronenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 10minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505nm. Activity of SGOT was expressed as IU/L.

## APPENDIX - 19

### ESTIMATION OF ALANINE AMINO TRANSAMINASE (AST)

#### Reitman and Franckel (1957)

SGOT catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate so formed is hydrazine derivative which is measured photometrically.



#### REAGENTS

1. Tris buffer, pH 7.5 – 100mmol/L
2. L- alanine – 500mmol/L
3. 2-oxoglutarate – 15mmol/L
4. 2,4, dinitronenyl hydrazine reagent
5. Working sodium hydroxide (4N)

#### Procedure

0.5 ml of buffered substrate was incubated at 37<sup>0</sup>C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37<sup>0</sup>C for 60 minutes. Then 0.5ml of DNPH reagent was added, mixed well and kept at room temperature for 20minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505 nm. Activity of SGPT was expressed as IU/L.

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**APPENDIX - 20****ESTIMATION OF MOISTURE CONTENT****Dry method (AOAC,1995).****Moisture content**

Moisture was determined using the drying method and the were reported on the dry weight basis. About 5 g of ground sample accurately weighed into a moisture dish was transferred into a hot-air oven previously heated to around 105°C and then drying was done for one hour. Final weight of sample was taken after drying and cooling in a desiccator. The sample residue is to be taken as the total solids and lose in weight as the moisture content of the sample was calculated

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**APPENDIX - 21****ESTIMATION OF ORGANIC CARBON****TITRIMETRY****(Walkley and Black, 1934)****PRINCIPLE**

Organic carbon present in organic matter is oxidized by chromic acid ( $K_2Cr_2O_7$ ) in the presence of concentrated  $H_2SO_4$ . This provides nascent oxygen which combines with carbon and form  $CO_2$ . The  $H_2SO_4$  enables easy digestion of organic matter by rendering heat of dilution. Only a certain quantity of chromic acid is used for oxidation. The excess chromic acid left by organic matter is determined by titration with 0.5N ferrous sulphate or ferrous ammonium using diphenylamine indicator.

**REAGENTS**

Potassium dichromate (1N), diphenylamine indicator (aqueous), ferrous sulphate solution (0.5N), concentrated sulphuric acid, orthophosphoric acid (85 %).

**PROCEDURE**

0.2g of sieved soil samples were transferred into a 500ml conical flask separately. 10ml of 1N potassium dichromate and 10ml of concentrated sulphuric acid solution was added, stirred well and kept for 30 minutes. To this, 200 ml of distilled water, 10ml of phosphoric acid and 1ml of diphenylamine indicator were added. The solution in the conical flask was titrated against 0.5N ferrous ammonium

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sulphate and the appearance of brilliant bright green was noted and the amount of organic carbon was calculated.

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## APPENDIX - 22

### DETERMINATION OF TOTAL DISSOLVED SOLIDS FILTRATION METHOD (APHA, 1998)

#### PRINCIPLE

A well-mixed sample is filtered through a standard glass fiber filter and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 18000. The increase in dish weight represents the total dissolved solids.

#### PROCEDURE

250mL of the effluent was filtered through a glass microfiber filter paper and the filtrate was evaporated in a tarred porcelain dish which was preheated at 105°C and then at 550°C for one hour in a muffle furnace. The porcelain dish was cooled and brought to constant weight. The dish was kept at 180°C for 1 hour, cooled and weighed. The increase in weight denoted the amount of total dissolved solids. The results were expressed as mg total dissolved solids per litre of the sample. The total dissolved solid present in the sample was calculated.

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## APPENDIX - 23

### DETERMINATION OF TOTAL SUSPENDED SOLIDS FILTRATION METHOD (APHA, 1998)

#### PRINCIPLE

A well-mixed sample is filtered through a weighed standard glass-fibre filter and the residues retained on the filter are dried to a constant weight at 103oC – 105oC. The increase in weight of the filter represented the total suspended solids. If the suspended material clogs the filter and prolongs filtration, it may be necessary to increase the diameter of the filter or decrease the sample volume.

#### PROCEDURE

250ml of the effluent was filtered through a tared filter paper by applying suction. The filter paper was washed with distilled water to remove the soluble salts

and dried the filter paper for one hour in an oven at 103°C-105°C. The increase in weight represents the amount of total suspended solids. The results were expressed as mg total suspended solids per litre of the sample. The suspended solids present in the sample were calculated.

## APPENDIX - 24

### DETERMINATION OF TOTAL SOLIDS

(APHA, 1998)

#### CALCULATION

Mg/l total solids (TS) = mg/l total suspended solids (TSS)+ mg/L total dissolved solids (TDS).

## APPENDIX - 25

### ESTIMATION OF TOTAL ALKALINITY

#### TITRIMETRIC METHOD

(APHA, 1998)

#### PRINCIPLE

When a sample containing carbonate and bicarbonate are titrated against the standard sulphuric acid, phenolphthalein loses its pink colour when half of the carbonate is converted to bicarbonate. Twice this value is a measure of carbonates present in the sample. To the colourless solution, a few drops of methyl orange is added and titrated against sulphuric acid till straw yellow colour changes to pinkish red colour. This value gives the amount of acid required to neutralize the bicarbonate originally present and that from the carbonates. By subtracting the first titre value from the second one, acid required to neutralise the bicarbonate originally present in the sample is obtained.

#### Reagents

1. Sodium carbonate solution 1N: 13.25g was dissolved in 250ml water.
2. Sulphuric acid 1N: 28ml of concentrated sulphuric acid was made up to a litre with distilled water.
3. Sulphuric acid 0.02N: Diluted approximate volumes of 1N sulphuric acid to prepare 0.02 N sulphuric acid 1.0mg CaCO<sub>3</sub>.

4. Phenolphthalein indicator: 500mg of phenolphthalein was dissolved in 50 ml of ethyl alcohol and 50 ml of distilled water. 0.02N sodium hydroxide solution was added dropwise until a faint pink colour appeared.
5. Mixed indicator solution: 20 mg of methyl red and 100 mg of bromocresol green were dissolved in 100 ml of 95% isopropyl alcohol.

## PROCEDURE

To 25 ml of the effluent equal volume of distilled water and a pinch of phenolphthalein indicator solution was added in a 250ml conical flask. Pink colour appeared which was then titrated with 0.02N sulphuric acid until the solution became colourless. Three drops of mixed indicator solution was added to the solution in which phenolphthalein alkalinity had been determined and titrated against 0.02N sulphuric acid. The colour was changed from emerald green to light pink. (If no pink colouration occurred, it indicated nil phenolphthalein alkalinity).

## APPENDIX - 26

### ESTIMATION OF TOTAL HARDNESS EDTA-TITRIMETRIC METHOD (APHA, 1998)

#### PRINCIPLE

Ethylene diamine tetra acetic acid and its sodium salts (EDTA) form a chelated soluble complex when added to a solution of certain metal cations. If a small amount of dye such as Eriochrome Black - T or calmagite is added to an aqueous solution containing calcium and magnesium ions at a pH of  $10.0 \pm 0.1$ , the solution becomes wine red. If EDTA is added as a titrant, the calcium and magnesium will be complexed and the solution turns from wine red to blue, marking the end point of the titration.

#### REAGENTS

1. Calcium standard solution: 1g of pure calcium carbonate was dissolved in 1 litre of distilled water using 20.5 ml of HCl and the contents were warmed.  
1 ml = 1 mg  $\text{CaCO}_3$ .

2. Standard EDTA titrant (0.02N): 3.723g disodium ethylene diamine tetra acetate dihydrate was dissolved in 1 litre of water. It was standardized against standard calcium solution. One ml of 0.02N EDTA 1mg of  $\text{CaCO}_3$ .
3. Eriochrome Black - T indicator: 0.5g of the dye was mixed with 100g of sodium chloride to obtain a dry powder mixture.

## PROCEDURE

50 ml of the effluent was placed in a conical flask, diluted to 50 ml and added. One millilitre of buffer solution was added per 50 ml volume of the sample. The pH of the titre should be  $10.0 \pm 0.1$ . A pinch of the indicator was added and titrated with standard EDTA, until a reddish tinge appeared. The end point was the appearance of blue colour.

## APPENDIX - 27

### ESTIMATION OF BIOCHEMICAL OXYGEN DEMAND

#### WINKLER'S LODOMETRIC METHOD

(APHA, 1989)

## PRINCIPLE

BOD determination involves the measurement of dissolved oxygen content of the sample, before and after 5 days incubation at  $20^\circ\text{C}$ . The reduction in oxygen content is due to the demand exerted by the microbiological population and it is a measure of oxidisable organic matter in the sample. When manganous sulphate is added to the sample containing potassium iodide, manganous hydroxide is formed, which is oxidized by the dissolved oxygen of the sample to basic manganic oxide. On addition of sulphuric acid, the basic manganic oxide liberates iodine, equivalent to that of dissolved oxygen originally present in the sample. The liberated iodine is titrated with a standard solution of sodium thiosulphate using starch as indicator.

## Reagents for the preparation of dilution water

1. Calcium chloride solution : 27.5 g was dissolved in one litre of distilled water.
2. Magnesium sulphate solution : 25 g was dissolved in one litre of distilled water.
3. Ferric chloride solution : 0.25 g was dissolved in one litre of distilled water.

4. Phosphate buffer (pH 7.2) 21.75 g dipotassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate heptahydrate and 1.7 g ammonium chloride were taken in one litre standard flask, dissolved in distilled water and made up to the mark. The buffer was stored in a refrigerator to prevent mold growth.

#### Reagents for the estimation of dissolved oxygen

1. Manganous sulfate solution : 91.9 manganous sulfate monohydrate was dissolved and diluted to 250 ml with distilled water.
2. Alkali - iodide - azide reagent Reagent A - 175 g potassium hydroxide and 37.5 potassium iodide were dissolved in 250 ml of water.
3. Reagent B - 2.5 g sodium azide was dissolved in 10.0 ml of water. Reagent A and B were mixed.
4. Concentration sulphuric acid.
5. Phosphoric acid : 85 - 90%.
6. Sodium thiosulphate solution (0.1 N): 24.82 g was dissolved in distilled water and made up to one litre.
7. Sodium thiosulphate solution, 0.025 N: 250 ml of 0.1 N sodium thiosulphate solutions was diluted to 1000 ml with distilled water.
8. 1 ml of 0.025 N sodium thiosulphate E 0.2 mg dissolved oxygen.
9. Starch solution - 1%

#### PROCEDURE

**Preparation of dilution water:** 1 ml each of calcium chloride, magnesium sulphate, ferric chloride and phosphate buffer solution was added to one litre of aerated distilled water and mixed thoroughly. This is the standard dilution water prepared freshly every time. **Seeding of the dilution water:** It is essential to seed the dilution water. The seeding material generally used is freshly settled raw sewage. 2 ml of raw sewage was added to one litre of dilution water.

**Dilution of the samples:** The test water samples were diluted with seeded dilution water sample (1%, 5% and 10%) in dilution mixture for the water sample. Each dilution sample was taken in a set of two BOD bottles.

**Determination of dissolved oxygen (DO) before and after 5 days incubation:** In one set of flasks DO was determined immediately while the other set was kept for

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incubation at 20° C for 5 days. After 5 days, DO for the incubated sample was determined. Determination of DO is as follows:

To the contents of the BOD bottle 2 ml of manganous sulphate solution and 2 ml of alkali - iodide - azide solution was added the bottle was stoppered and mixed thoroughly. A brown precipitate of basic manganic oxide was formed, which was allowed to settle completely leaving a clear supernatant liquid. Then 2 ml of conc. sulphuric acid was added along the sides of the bottle, stoppered and mixed for complete dissolution. The contents were transferred to a 500 ml conical flask and titrated immediately against 0.025 N sodium thiosulphate using starch as an indicator and BOD was calculated.

## APPENDIX - 28

### ESTIMATION OF CHEMICAL OXYGEN DEMAND

#### TITRIMETRIC METHOD

(APHA, 1998)

#### PRINCIPLE

Chemical Oxygen Demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. Because of its unique chemical properties, the dichromate ion, the specified oxidant is reduced to the chromic ion ( $Cr_3$ ).

COD often is used as a measurement of pollutants in waste water and natural waters. Most types of organic matter are oxidized by boiling the mixture of chromic and sulfuric acids. A sample is refluxed in strongly acid solution with a known excess of potassium dichromate. After digestion, the remaining unreduced potassium dichromate is titrated with ferrous ammonium sulfate to determine the amount of potassium dichromate consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

#### REAGENTS

1. Mercuric sulphate crystals.

2. Sulphuric acid - silver sulphate reagent: Dissolved 10.1 g of silver sulphate in 1 litre of concentrated sulphuric acid and allowed the solution to stand for two days for complete dissolution.
3. Potassium dichromate solution 0.125N: Dissolved 0.129 g of potassium dichromate in distilled water and made up to 1 litre. 1 ml of 0.125N potassium dichromate 1 mg of oxygen.
4. Ferroin indicator solution: Dissolved 95 mg of ferrous sulphate in 500ml of distilled water, added 1.485g of 1, 10 phenanthroline monohydrate and mixed thoroughly.
5. Ferrous ammonium sulphate solution 0.125 N: 40g of ferrous ammonium sulphate was dissolved in distilled water, 20ml of concentrated sulphuric acid was added and made up to one litre with water. The contents were standardized with 0.125 N potassium dichromate.

## PROCEDURE

A refluxing flask of 250 ml capacity was used with a ground glass 24/40 neck fitted with a 300 mm double surface condenser to which a glass cap was fitted. 50 ml of the sample was placed in the flask and a suitable quantity of mercuric sulphate was added so that the ratio of chloride content of the sample to mercuric sulphate was 1: 10 (For this, chloride content of the sample was estimated). Five ml of sulphuric acid - silver sulphate reagent was added to dissolve the mercuric sulphate and cooled in cold water while mixing. 25ml of 0.125 N potassium dichromate was pipetted into the flask and mixed well. 70 ml of sulphuric acid - silver sulphate reagent was added very carefully and mixed. A few porcelain bits were added and the condenser was attached to the refluxing flask. Water was circulated and refluxed for two hours. The flask was cooled after removing from the flask. The contents of the flask were transferred and diluted to 350ml with distilled water. About 2 to 3 drops of ferroin indicator was added and titrated against 0.125N ferrous ammonium sulphate solution. The end point was the sharp colour change from blue-green to reddish brown. A blank was conducted using 50ml of distilled water instead of the sample and COD was calculated.

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**APPENDIX - 29****ESTIMATION OF CHLORIDES  
SILVER NITRATE TITRIMETRIC METHOD  
(Vogel, 1964)****PRINCIPLE**

Silver nitrate reacts with chloride ions to form silver chloride. The completion of reaction is indicated by the red colour produced by the reaction of silver nitrate with potassium chromate solution which is added as an indicator.

**REAGENTS**

Chloride free double distilled water was used for all reagents.

1. Standard silver nitrate titrant, 0.0282N: 4.791 g of silver nitrate was dissolved in 1 litre of distilled water. Standardized it against 0.0282 N sodium chloride solution. 1 ml of exactly 0.0282 N AgNO<sub>3</sub> E 1.0mg chlorides.
2. Standard sodium chloride titrant, 0.0282 N: 1.648 g of sodium chloride was dissolved in 1 litre of distilled water. 1.0 ml mg chloride.
3. Potassium chromate indicator solution: 25 g of potassium chromate was dissolved in 100ml of distilled water and silver nitrate solution was added dropwise until a slight red precipitate was formed. The contents were allowed to stand for 12 hours, filtered and made up to 500ml with distilled water.
4. Aluminium hydroxide suspension: 100 g of aluminium ammonium sulphate was dissolved in 1000 ml distilled water and warmed to 60°C by stirring 55 ml of conc. ammonia solution.
5. The precipitate was allowed to settle for an hour and washed with distilled water to make the precipitate free from chloride. The decantate was checked by treating a portion of it with silver nitrate solution until the precipitate was free from chloride and diluted it to 1000 ml with distilled water.

**PROCEDURE**

To 50 ml of the effluent, 3 ml of aluminium hydroxide was added, stirred well, allowed to settle and filtered. The precipitate was washed with chloride free distilled water, the filtrate and washings were combined. 100ml of the samples were pipetted into a porcelain dish and the pH was adjusted in the range of 7 - 9.5. One ml of

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potassium chromate indicator solution was added and titrated it against standard silver nitrate solution with constant stirring until a slight precipitable reddish colouration persisted. A blank was measured by placing 100 ml chloride - free distilled water instead of sample and total chloride was calculated

## APPENDIX - 30

### ESTIMATION OF SULPHATES TURBIDIMETRIC METHOD (APHA, 1998)

#### PRINCIPLE

Sulphate ions are precipitated as barium sulphate crystals of uniform size in acid medium. Light absorbed by the precipitate is measured using a spectrophotometer.

#### REAGENTS

1. Conditioning reagent: 75 g of sodium chloride was dissolved in 300 ml distilled water, added 30 ml conc. HCl and 100 ml of 95% ethyl alcohol (or isopropyl alcohol). To the above, 50 ml of glycerol was added and mixed well.
2. Barium chloride solution: 100 g of barium chloride was dissolved in 1000 ml of distilled water and was filtered through Whatman No.1 filter paper.
3. Standard sulphate solution: 147 mg anhydrous sodium sulphate was dissolved in distilled water and made up to 1 000ml. 1 ml = 100 $\mu$ g SO<sub>4</sub><sup>2-</sup>

#### PROCEDURE

##### Standards

In a 250 ml conical flask 5, 10, 15, 20, 25, 30, 35 and 40 ml of standard sulphate solution was measured separately and diluted to 100 ml. 5 ml of conditioning reagent was added and mixed well using magnetic stirrer. The speed of stirring should be the same for both standards and samples. While stirring, 0.5 g of barium chloride crystals was added and stirring was continued for one minute. Immediately after one minute, an aliquot of the solution was taken in an absorption cell and measured the optical density at a wavelength of 420 nm. A blank was carried out and a graph was plotted relating optical density to  $\mu$ g of SO<sub>4</sub>.

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**SAMPLES**

To a 250 ml conical flask, 50 ml of the sample was measured and diluted to 100 ml and preceded from steps as for standards. From the calibration graph, the mg of sulphate equivalent to the optical density was calculated.

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**APPENDIX - 31****ESTIMATION OF NITRATES****COLORIMETRIC METHOD****(APHA, 1998)****Reagents: nitrate stock solution**

722 mg potassium nitrate was dissolved in distilled water and made up to 1000 ml in a volumetric flask.

**Nitrate standard solution**

100 ml nitrate stock solution was pipetted in to a 1000 ml volumetric flask and made up to the mark with distilled water.

**Brucine – sulphanilic acid solution**

1 g brucine sulphate and 100 mg sulphanilic acid was dissolved in 70 ml hot distilled water 3 ml conc. HCl was added cooled and diluted to 100 ml with distilled water.

**Sulphuric acid solution**

500 ml conc.  $H_2SO_4$  was added to 75 ml distilled water and cooled to room temperature.

**PROCEDURE**

1. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml of nitrate standard solution were taken in a series of 50 ml beakers and diluted to 5 ml with distilled water.
2. A beaker containing 5 ml of distilled water was used as a blank.
3. 2 ml of the sample was taken in a 50 ml beaker and diluted to 5 ml with distilled water. 1 ml of brucine sulphanilic acid solution was added to the blank, standards and sample are mixed well.

4. 10 ml of Sulphuric acid solution was taken in a second series of 50 ml beakers. The contents of the first series of beakers were poured in to each of the second series of beakers and mixed well. Beakers were kept in the dark for 10 minutes.
5. 10 ml of distilled water was added to all the beakers. Beakers were allowed to cool for 20 – 30 minutes. The colour development was read in a colorimeter against 510 nm. Using the calibration curve the mg equivalent of nitrate nitrogen in the sample was calculated.

### APPENDIX - 32

#### Authentication certificate for algal identification



भारत सरकार  
GOVERNMENT OF INDIA  
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय  
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE  
भारतीय वनस्पति सर्वेक्षण  
BOTANICAL SURVEY OF INDIA



दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre  
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सं. भा.व.स.द.क्षे.के./No.: BSI/SRC/5/23/2018/Tech.

/2639

दिनांक/Date: 31<sup>st</sup> December 2018

#### पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The algal specimen brought by you for authentication is identified as *Oedogonium* sp. - OEDOGONIACEAE. The identified algal specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

डॉ सी मुरुगन/Dr. C. Murugan  
वैज्ञानिक 'ई' एवं कार्यालय अध्यक्ष /  
Scientist 'E' & Head of Office

सेवा में /To

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